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Induced fucosyltransferase gene expression in Zika virus infected human cells

Degree Project in Medicine

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Table of contents

Table of contents 2
Abstract 3
Introduction 5
History and Virology of the Zika Virus 5
Transmission of the Zika virus 6
Clinical aspects of the Zika virus 7
Flavivirus and their genetics and structures 10
Glycoproteins, Glycosylation and Fucosyltransferases 12
Glycovirology and the potential roles of glycans in Flavivirus 14
Methods and material 18
Cellular infection 18
Cellular harvesting and RNA- extraction 19
Quantitative reverse transcriptase polymerase chain reaction (qRT- PCR) 19
Analysis method 22
Ethics 23
Results 24
Time course of Zika virus RNA content in infected HUM and A549 cells 25
Gene expression of FUT2 and FUT4 in A549 cells and in HUM cells 26
Gene expression of FUT1, FUT3, FUT5, FUT6 and FUT7 showed no difference between Zika infected and mock infected cells 27
Discussion 29
Acknowledgments 31
Populärvetenskaplig sammanfattning 32
References 34

Abstract

Introduction: The Zika virus (ZIKV) has in the 2010's emerged as a new mosquito- borne virus that causes a threat to the public health, especially due to the rise of microcephaly seen. As with other Flaviviruses, the E-protein is of great importance for infectivity, and recent mutations have led to the dramatic rise of infectivity and complications. The polysaccharides constituting the glycan part of the glycoprotein are unknown, as is the mechanism of viral spread and how it gets into the central nervous system. Fucosyltransferase is one glycosyltransferase involved in synthesizing polysaccharides adding fucose to glycans.

Aim: Our aim was to investigate the expression of the fucosyltransferase genes 1-7 in ZIKV-infected human cells.

Methods: Human cell lines A549 cells and HUM cells were grown and infected by the Uganda strain of the ZIKV. Quantitative reverse transcriptase PCR (qRT- PCR) for fucosyltransferase 1 – 7 mRNA analysis was conducted on infected cells and mock- infected cells.

Results: The expression of fucosyltransferase gene 4 in ZIKV- infected A549 cells was increased 48 hours post- infection (P 0.00035) and 72 hours post- infection (P 0.00014). The expression of fucosyltransferase gene 2 was increased 72 hours post- infection in ZIKV-infected A549 cells, however this increase was not significant.

Conclusions: We have demonstrated an increase in expression of the fucosyltransferase 4. The result of the increased expression is unknown. Fucosylated glycans are known

to be used by viruses for viral spread, such as the case with HTLV-1. Further studies need to focus on unravelling the structure of the E-protein; its glycans and functions.

Keywords: Zikavirus, ZIKV, fucosyltransferase.

Introduction

History and Virology of the Zika Virus

The Zika virus was first discovered in Uganda in 1947 but for a long period did not receive much attention since there had not been any greater outbreaks. This changed when in 2013 and 2014 outbreaks in French Polynesia and Easter Island occurred, followed by Brazil in 2015. As serious neurological symptoms such as Guillain- Barré syndrome and congenital malformation during pregnancy began to be linked with ZIKV, extensive research have been performed (1). At first, the Zika virus was not believed to be causing human disease. The first cases were reported in Nigeria in 1953 where three cases were noted (2). During the next 57 years only 13 cases were to be reported. The first bigger outbreaks occurred in Micronesia, resulting in 5000 infected in the State of Yap in 2007, and later involving an estimation of 32 000 infected in French Polynesia in 2013 – 2014 (3).

The evolution of ZIKV begins in Africa and then split into two major lineages - one African and one Asian/American. The African lineage consists of two groups - one Uganda cluster, where the prototype MR766 strain belongs and one Nigeria cluster. The Asian/American lineage consists of one strain isolated in Malaysia with the prototype P6-740 strain, which has also been found in Cambodia, Micronesia and French Polynesia. From this strain the American lineage emerged and introduced the virus into the Western Hemisphere with the big outbreak in Brazil in 2015 (4). The Brazilian Ministry of Health has estimated 440.000-1.300.000 persons to have been infected during the outbreak (5). Because of the big outbreak

and the increased number of microcephaly and Guillain- Barré Syndrome, the World Health Organization declared the Zika outbreak to be an emergency of public health concern (6). The emergence of neurological complications, such as microcephaly in fetus in Zika virus infected pregnant women , could be explained by the higher viremia through adaptive evolution, resulting in transplacental transmission and or changes in cell tropism in the Asian/American strain. The higher viremia has also led to enhanced transmission and spread. Additionally, the acquired mutations in the newer strain has led to enhanced infection of midgut epithelial cells in mosquitos enabling wider spread (4).

Transmission of the Zika virus

As in the case with most other Flavi virus, the main spread of the virus is made by an arthropod. The Zika virus is spread by different mosquito species.
In non- urban areas the virus is being spread between nonhuman primates and occasionally to humans by different *Aedes* mosquitos, primarily species belonging to the subgenera of *stegomya* and *diceromya*. In urban and suburban areas, the transmission is in a human-mosquito- human cycle, by the *Aedes* species *aegypti* and *albopictus* (3). These are also the most common mosquitos to spread the Yellow fever and Dengue virus (7).
Both *A. aegypti* and *A. albopictus* are widely distributed in the Americas and the rest of the tropical and subtropical world. *A. albopictus* can be located to more temperate areas, thus extending the potential range away from the equator. Both species bite primarily during daytime. *A. Aegypti* mosquitos have an especially high ability to transmit pathogens to humans. This is due to its close association with human habitation. In fact, human blood is its main food source and it often bits multiple humans in a single blood meal, enhancing transmission (3).

Although the main transmission route is via mosquitos, there exist to some extent other ways of transmission. The infection of a fetus in a Zika virus infected pregnant woman is due to a transplacental spread. Sexual transmission of the virus has also been confirmed. According to CDC, 14 cases of suspected sexual transmission have been reported, where a male partner has been infected abroad and transmitted it during sexual intercourse to a partner. Viral RNA in sperm in Zika virus infected men have been confirmed (8).

Also, during the Zika virus outbreak in French Polynesia 3 % of donated blood samples tested positive for Zika virus and although no case of transmission through blood transfusion has been reported, this is a likely possibility (9).

Clinical aspects of the Zika virus

Far from everyone infected by Zika virus develops symptoms. In the Yap outbreak 19 % of everyone showing positive seroprevalence of Zika virus had symptoms consistent with Zika virus symptoms (10). The symptoms, which occurred at a similar frequency in the Yap outbreak and in pregnant women in Brazil, include a macular or papular rash (90 % of patients), a short- term and low grade fever (65 %), arthritis or arthralgia (65 %), conjunctivitis (55 %), myalgia (48 %), head ache (45 %), retro- orbital pain (39 %), edema (19 %) and vomiting (10 %) (10, 11). The time between infection and symptoms is unknown but is probably in line with other mosquito- borne flaviviruses where the incubation time is less than a week. A volunteer showed symptoms after 82 hours incubation period, after being inoculated with the Eastern Nigerian strain of the Zika virus (12).

Neurological complications were soon reported in the outbreaks of the Zika virus. Primarily Guillain- Barré syndrome (GBS) has been linked with Zika virus, but also cases of meningoencephalitis and acute myelitis have been reported (3). A case- control study in French Polynesia during the outbreak in 2013 showed a strong association (odds ratio > 34) between Zika infection and GBS. In that study 93 % of 42 patients diagnosed with GBS had anti-ZIKV IgM and 88% of the patients reported symptoms compatible with ZIKV prior to GBS (13).

ZIKV has also been described in other cases of neurological complications. One case of meningoencephalitis has been described in a 81- year old man where the only positive test in the cerebrospinal fluid was for ZIKV (14).
Another described neurological complication is a case of acute myelitis in a 15-year old girl with positive ZIKV in the blood and cerebrospinal fluid (15).
The total extent of cases of meningoencephalitis and myelitis is unknown.

The teratogenic effects by the Zika virus are probably the most associated effects known by the public. The total extent of the impact on fetal development is unknown. Microcephaly, the most commonly reported fetal complication, is a clinical finding of a small head size, indicating a problem with the brain growth. According to the guidance from Centers for Disease Control and Prevention, microcephaly is to be diagnosed when the occipitofrontal circumference is below the third percentile for gestational age and sex (16). When this guidance is followed, about 6 cases per 10 000 live births are born with microcephaly in USA (3).

Evidence for an association with the ZIKV consist of the fact that Zika virus RNA has been found in the amniotic fluid and in the brain tissue of fetuses and infants with microcephaly (17) and the high rate of microcephaly in children bore by mothers that had been infected by Zika prior to birth, and primarily in their first trimester (3).
The extent of cases of microcephaly is not completely clear. By June 2016, 7830 suspected cases had been reported to the Brazilian Ministry of Health. A review of the 1501 suspected cases in liveborn babies with microcephaly concluded that 602 cases were likely attributable to Zika virus infection (18). Since the majority of ZIKV infections does not cause any symptoms, the precise number of infected is difficult to predict. It is also unclear if the rate of fetal anomalies is the same in pregnant women presenting symptoms than in those not presenting any. In a cohort study where 88 pregnant women had an acute episode of rash, 82 % were infected by ZIKV and in which 42 % fetal abnormalities were found. 3,4 % of the infants born by infected mothers were born with microcephaly (11).

Aside from the small brain volume, ZIKV infection is likely to cause directly brain cellular injury, which is seen as intracranial calcification probably related to cell death leading to disruption of existing immature neurons and decreased proliferation. These intracranial calcifications can be visualized using fetal ultrasonography and are mainly located subcortical leading to thin cerebral cortices (17).

Other anomalies often found in infants with microcephaly and confirmed prenatal ZIKV infection are microphthalmia, coloboma, cataracts and intraocular calcifications. These ocular anomalies are reported in 24- 55 % of infants with ZIKV associated microcephaly (19).

Since there does not exist any vaccine nor treatment, the means of control are limited to reducing the breeding sites of *the A. aegypti* and minimizing the risk of getting bitten through the usage of mosquito repellent, window screens etc. (3).

Flavivirus and their genetics and structures

The name Flavi derives from the Latin word flavus, which means yellow, hinting at one member of the family – the Yellow fever virus, that may cause jaundice. The common method of transmission for many Flaviviruses is via arthropods, either via mosquitos as in the case with Zika virus (ZIKV), West Nile virus (WNV), Japanese encephalitis virus (JEV), Dengue virus (DENV) and Yellow fever virus (YFV) or via ticks as in the case with tick- borne encephalitis virus (TBEV). Dengue virus causes the highest rates of disease and mortality among Flavivirus, where hemorrhagic fever can evolve in a person that has been infected sequential by different serotypes. Currently, there are available vaccines for Yellow fever virus, Japanese encephalitis virus and Tick- borne- encephalitis virus (20).

Flavivirus is a single- stranded, positive sense RNA- virus. The genome is 10.6 kb large and encodes one single polyprotein, which upon cleavage by different proteases results in one capsid protein, one membrane protein (prM), one envelope protein (E protein) and seven non-structural proteins (20).

The capsid protein forms the nucleocapsid core, which is one of the earliest events in the assembly of flavivirus, and it is also involved in packaging of the viral genome. The genomic

RNA is enclosed by capsid proteins, forming the nucleocapsid. Upon entrance into the cytoplasm of the host cell, the nucleocapsid disassembles (20).

The M-protein is a non- functional rest of the precursor protein prM, which is cleaved when the immature virion is budding into the ER by the host protease furin. With the prM still in the envelope the E-protein cannot function (20). This is due to its function as a chaperone for folding and assembling the E protein. While prM is still present in the envelope, the E-protein is not dimerized and cannot perform the needed conformational changes that take place in response to the acidic environment a cell membrane means (21).

The E-protein is engaged in receptor- binding and fusogenic activities. The E-protein in a mature virion is dimerized, with the fusion loop buried in the link of the two dimers. The fusion loop interacts with the target membrane upon dissociation of the dimer, which appears in response to lowered pH in presence of a membrane, enabling virus entry (22)

The E-protein of the Asian/American strain has one N- linked glycosylation site where N-glycosylation must take place to create a functional E-protein. This novel N- glycosylation site is at residue Asn- 154 of the E-protein, acquired through mutation, is absent in the older strains. The increased virulence and pathogenicity seen in the Asian/American strain is linked to this mutation. The new N-glycosylation site is also probably accountable for the neurotropism seen in the more recent outbreaks (23, 24).

All parts of the E-protein are not completely conserved throughout the Flaviviridae, with about 40 % of the amino acids being identical. The structural differences between E-protein among flavivirus might be the responsible structure for the different cellular tropism.

Beside the structural proteins C-, prM- and E- protein the polypeptide also contains the non-structural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5, which are important for entry, translation, replication, morphogenesis and pathogenesis (20).

Flavivirus are enveloped, consisting of a lipid membrane originating from the host cell and glycoproteins. The infection starts with viral membrane fusion facilitated by viral E-protein. (1). After the receptor- mediated endocytosis has taken place, the nucleocapsid disassembles and the genome is being released into the cytoplasm. As the viral genome is a single- stranded positive sense RNA, it can without intermediate steps be translated in the host cell to a polyprotein. The polyprotein is then co- and post- translationally processed by host cell and viral proteases, resulting in the viral proteins. Immature virions are assembled in the lumen of the host ER by the translated structural proteins and newly synthesized RNA. The replication of the viral RNA takes place on intracellular membranes. The immature virion is turned into a mature one after the transport through the trans- Golgi network where host protease furin cleaves prM into M- protein. The mature virions exit the cells by exocytosis (20).

As with other Flaviviruses, the ZIKV has tropism for the central nervous system since it can cause neurological disorders, but the exact mechanism behind this is unknown as is the way of transport in to the central nervous system, In vitro studies have shown that ZIKV can infect embryonic brain cells and cranial neural crest cells; cells that are crucial in facial bones development (25).

Glycoproteins, Glycosylation and Fucosyltransferases

To understand the examined fucosyltransferase genes and how they could be of viral interest we first need to look at their function and how polysaccharides are synthesized.
Glycans are polysaccharides that consist of many monosaccharides, which are linked together glycosidically. Glycosyltransferases are the enzymes responsible for establishing the glycosidic bonds between the monosaccharides that make up the glycan (26).
Glycoproteins are made of a glycan attached covalently to a protein through a glycosidic bond. The glycosidic bond between the glycan and the protein is either made by a N-glycosylation process (making a N- linked glycan) or an O- glycosylation process (making a O- linked glycan). In the N- linked glycan the glycosidic bond links a carbohydrate to a nitrogen- molecule on the side chain of the amino acid asparagine, and in the O- linked glycan the glycosidic bond links a carbohydrate to an oxygen molecule on the side chain of serine or threonine
The glycosylation process takes place in the cell after the translation of the protein. N-glycosylation begins in the ER and continues in the Golgi, whereas O- glycosylation only takes place in the Golgi complex. (26).

Fucosyltransferases is one group of glycosyltransferases, that catalyze the transfer of fucose from the nucleotide sugar Guanosine 5'- diphospho-beta- L- fucose to galactose and to N-acetylglucosamine. The transfer of fucose by a fucosyltransferase is called fucosylation. The usage of fucosyltransferases are widely distributed throughout the eukaryotes and prokaryotes.
There are twelve human fucosyltransferase genes (1 to 11 and polypeptide O-fucosyltransferase 1 and 2), and their function vary in the recognition of the acceptor and in the type of linkage formed (27, 28). The fucose is transferred by a fucosyltransferase to

various acceptor molecules. These include oligosaccharides, glycolipids and glycoproteins of both N-glycan and O- glycan type.

In mammals fucosylation is involved in the last steps of synthesis of the A, B and H Lewis blood antigens, as well as Lewis- related carbohydrate antigens, like sialyl- Lewis^x (sLe^x), Lewis^x (Le^x) and Lewis^y (Le^y). Fucosyltransferase 4 and 9 is essential for synthesis of Le^x. Biosynthesis of sLe^x is made by fucosyltransferase 3,5,6 or 7, of which 6 and 7 have the highest potency (29). Fucosyltransferase 4 and 7 are expressed in leukocytes, crucial for synthesizing selectin ligands which upon binding to endothelial selectins initiate the adhesion between leukocyte and endothelial cell. Thus, these are necessary for normal leukocyte trafficking and function (29, 30).

Some of the fucosyltransferase genes are, however, not essential for normal biological processes in the human body; number 3, 5 and 6 (29).

Glycovirology and the potential roles of glycans in Flavivirus

The interest in glycans and their role in many biological processes, has raised the concept of glycomics and the special importance of glycans in the host- virus interaction has led to a concept termed glycovirology. Some viruses are known to be able to affect the expression of host glycosyltransferases to produce viral glycans in the host ER and Golgi apparatus. The virus uses the glycans to for example avoid host anti- viral mechanisms or to alter the host metabolism (31). One example of glycosylation's role in infectivity is the glycoprotein hemagglutinin of the influenza virus that interact with glycans on the host cell which is crucial for virus entry (32).

Enveloped viruses, such as the Zika virus and other Flavivirus, possess an envelope derived from the host cell, interspersed with viral proteins glycosylated by the host cell. The glycosylation of the Flavi E-protein has been repeatedly demonstrated to be necessary for infectivity. As earlier mentioned, the older African strains of the Zika virus in opposite to the newer strains responsible for the recent outbreaks, do lack an N- linked glycosylation site in the E- protein (23). Experiments where mice were infected by recombinant ZIKV of the newer strain lacking that N- linked glycan, showed heavily reduced neuroinvasion. Mice that were infected with ZIKV containing the N- linked glycan died, whereas mice infected with the recombinant ZIKV lacking the N-linked glycan survived and showed much lower levels of ZIKV RNA in their brain tissue. However, mice that were infected with the ZIKV lacking the N-linked glycan intracranially showed similar lethality and infection signs as the ZIKV with the N- linked glycan. This speaks for the importance of the N- linked glycan in enabling the virus a transport through the blood- brain- barrier but not necessarily for neuro- infectivity itself (33).

Experiments conducted on the West Nile virus, another mosquito- borne flavivirus, showed that mutated virus lacking the N-linked glucan on the E- (envelope) and NS1- (non-structural 1) proteins, were much less neuroinvasive compared to the normal virus. Attenuation was seen in mice infected by WNV with a mutation in the NS1 protein. No signs of neuroinvasiveness were seen in mice infected by WNW with mutations in both the NS1 protein and in the E-protein. (34).

Experiments in which mice were infected with recombinant tick- borne encephalitis virus (TBEV), where the N- linked glycosylation site on E- protein was removed, did not show any signs of infection. In mammalian cell cultures, the mutated virus leads to reduced secretion of infectious virions, which is consistent with the results in mice. (35).

All these studies have demonstrated the crucial role the N-linked glycosylation of the E-protein of the Flavivirus has for infectivity. Less is known of what the glycan structures of the E- protein can bind to. DC-SIGN is one of few known receptors to which the Flavivirus can bind. DC-SIGN is known to be recognizable by the E-protein of Dengue- virus and West-Nile virus. Once these viruses are injected in the skin by a mosquito, dendritic cells can thus get infected, being the first step in the infection. Dengue virus infectivity was inhibited when incubating with anti- DC-SIGN antibodies (36, 37). Zika virus has also been demonstrated to be able to bind to DC-SIGN. In a study Zika virus infection was increased in cells normally not being infected upon induction of DC-SIGN expression (38). DC-SIGN is a cell- surface receptor expressed on dendritic cells. The receptor binds preferentially to oligosaccharides that are either high- mannose type or that are having a terminal fucose linked with galactose. Examples of such oligosaccharides are Lewis antigens Le^a, Le^b, Le^x and Le^y (39).
Dendritic cells are monocytes that have migrated to tissues and differentiated to an antigen-presenting cells. When a pathogen has been found, the cells migrates to secondary lymphoid organs and present it to T- cells. This procedure is believed to be used in HIV-1 infection, where the HIV-1 glycoprotein gp120 binds to DC-SIGN, enabling transport of the virus to CD4+ T- cells in secondary lymhoid organs with the help of dendritic cells (40).

The purpose of our study was to investigate whether the expression of fucosyltransferase genes was increased in Zika virus- infected cells. As the N- glycan part of the E- protein is of high importance in viral spread and pathogenesis and the constituting glycans are unknown, expression of fucosyltransferases could provide information regarding the potential outer structures of the N- glycan. Knowledge of the pathogenesis is of great importance in understanding the mechanism of neuroinvasiveness seen in the South American strain. Understanding the mechanism is a fundamental step in developing treatment and or

vaccination of Zika virus infection.

Methods and material

Cellular infection

Human embryonic cells (HUM- cells) and A549 (human adenocarcinoma alveolar basal epithelial cells) were allowed to grow densely in bottles for 24h before ZIKV infection. Three batches (biological replicates) of each cell culture were made.
The medium used for both cell cultures destined to be infected with ZIKV contained 2 % DMEM (Dulbecco's Modified Eagle Medium, Waltham, MA), fetal calf serum and 1 % PEST (Penicillin- Streptomycin 10.000 U/mL, Thermofisher, Waltham, MA) and distilled water to cover the cell cultures.

The cell cultures were distributed on a 12- well plate for growth. Thereafter the cell cultures were infected by the Uganda strain Zika virus (U976) and incubated at 37 degrees for two hours in a CO_2 incubator. The number of virions added to the wells, equaled the number of cells, hence the multiplicity of infection (MOI) was one. Half of the cells were however not infected, destined to be control cells. These cells were mock- infected, hence handled in the same manner as virus- infected cells, but without a virus being injected.

The handling of the virus and the infection of the cells were previously performed and took place in a P3 safety lab.

Cellular harvesting and RNA- extraction

The cell cultures were previously extracted and had been harvested by removing the medium and adding 350 microliter Qiagen RNeasy (Hilden, Germany) a lysating buffer that lysate the cell membrane. This procedure occurred at different times to allow different viral infection time, 0 hours, 24 hours, 48 hours and 72 hours post- infection. To create favorable conditions for RNA binding to the RNeasy membrane, 350 microliters ethanol was added. The samples were centrifuged, binding the total RNA to the RNeasy membrane and washing the contaminants away. The total RNA was eluted twice in RNase- free water.

The RNA concentration of the samples was analyzed using Nano- drop technique and diluted to a concentration of 20 nanogram/microliter to allow the qRT- PCR to perform at the highest precision.

Quantitative reverse transcriptase polymerase chain reaction (qRT- PCR)

The PCR method is based on cyclic temperature changes that allow different reactions to take place. At the highest temperature, 95° C, the DNA strands are separated. When the temperature lowers to 45°C, added specific primers to the sample can bind to the 3´and the 5´end of the separated DNA. The temperature is thereafter raised to 72°C where the Taq polymerase will extend the DNA strand at the ends of it by incorporating nucleoside triphosphate. At the end of the cycle a copy of the original DNA has been produced. After all cycles have been completed in a conventional PCR, the user will compare the results of the agarose gel to that of a known DNA ladder and will find out if the wanted DNA strand existed.

Quantitative PCR (qPCR), also called real-time PCR, made it possible not only to detect a certain gene, but also made it possible to distinguish the relative amount of the gene. Since the quantification and detection could occur simultaneously the initial amount of genetic material now made a difference. Basically, the method of qPCR is the same as in PCR, however to get the results in real time a fluorescent probe per gene of interest is being added. Commonly, a 96- well plate is used, allowing many samples to be analyzed simultaneously.

The amount of fluorescence being emitted by the sample is proportional to the amount of DNA strands that has been synthesized. At first, when there are a few replicates of the gene of interest, the fluorescent signal is not more powerful than the background signal. When the quantification continues, the signal strengthens and will at first increase exponentially. Eventually, the exponential increase is being replaced by a linear one, which represents a phase where the quantification is being limited by depletion of primers, fluorescence or nucleotides in the sample. The point where the saturation is reached is usually the same for all the samples, regardless of the initial DNA amount. This differs from PCR, where the end point is the point of interest. In qPCR the numbers of quantification cycles, C_T - value, needed for the fluorescence to reach a certain threshold is of interest. In a sample where the DNA of interest is occurring in high levels, the numbers of quantification cycles will be less needed than in a sample where DNA of interest is lower, resulting in a lower C_T – value. Thus, the lower the C_T – value, the higher amount of initial DNA in the sample.

The C_T – value is an arbitrary unit, which will be different in different experiments. In order to calculate threshold levels that can be compared to those calculated in other experiments, usage of a series of diluted DNA strands needs to be used.

Since the genetic material of interest in this study was RNA and not DNA, reverse transcriptase was added to the samples to synthesize a complementary DNA strand (cDNA). The reverse transcription can proceed with or without a primer, the latter resulting in lower

efficiency. The primer can be gene specific or bind to the 3´ poly- A- tail of the mRNA and start the reverse transcription from the beginning of the RNA strand. However, if the gene of interest is located far away from the poly- A- tail, there is a risk of that sequence not getting transcribed. Another possibility is random sequence primers, which are short strands of all possible sequences.

In our case, we have used a specific primer that is both primer for the reverse transcriptase and for the Taq polymerase, since we have used one step qRT-PCR. This is possible since we only analyzed one gene at a time. The primers used were specific to the fucosyltransferase 1-7 genes. The fluorophore, which is bind to a DNA- probe is also gene- specific. The probe binds to one of the strands during the low temperature- phase. In the high temperature phase the Taq polymerase, which also exhibits endonuclease activity, will cleave the DNA- probe. This will bring the fluorophore in an excited state; thus emitting light.

For determination of the amount of cells, the expression of the housekeeping gene RPL4 which is directly proportional to the number of cells and unaffected by infection was determined by qRT-PCR.

In all the experiments, the procedure and qRT-PCR settings were identical. First, a mastermix containing Superscript® III Platinum® one- step qRT-PCR kit with Rox (Invitrogen by Life technologies), gene specific primers and probes, reverse transcriptase and RNase free water were mixed together and distributed on a 96- well plate. Second, known concentrations (10, 1, 0,1 and 0,01 picogram/microliter) of the gene of interest inserted in plasmids were added in the top row of the plate. Third, the samples of the RNA from the cell cultures containing unknown concentration of the gene were added. Samples were taken from the three batches respectively and of every sample a triplicate was analyzed. Fourth, a negative control, pure water, was added in one of the wells. Fifth, a short program centrifugation of the samples (1000 rpm, one minute), was performed. Sixth, the qRT-PCR program was executed in a

Quantstudio5 (applied biosystems by thermo Fischer scientific) using 30 min pre- warming at 50ºC for reverse transcription, and the standard protocol (95ºC and 60ºC, 40 cycles).

Table 1. The table shows the different primers and probes used in the analysis. Acc. Number refers to the accession number in the NCBI Database.

Gene	Acc.Number (mRNA)	Forward Primer	Reverse Primer	Probe
FUT1	NM_000148	AGGTATAAACACACCCTCTGTGCTT	GAGTTCAGGGACAGACAGTGGTT	AAACTGGCAGGTACCGTGCTCATTGC
FUT2	NM_000511	CTCGCTACAGCTCCCTCATCTT	CGTGGGAGGTGTCAATGTTCT	TGGTCACCAGTAATGGCATGGCCTG
FUT3	NM_000149	GGGATCCCTTTTCGTCACACT	CGAACTGGTCTAAGCCTTGCA	AGGTGACCTACAGGCTCCGCTCGA
FUT4	NM_002033	AATTGGGCTCCTGCACAC	CCAGGTGCTGCGAGTTCT	TGGCCCGCTACAAGTTCTACCTGG
FUT5	NM_002034	CGCTGGATCTGGTTCAGCAT	CAGCCGTAGGGCGTGAG	CCCCCAGCAACTCCGGC
FUT6	NM_000150	GCATCCAGACGGGATCCA	ACTGCTGCGTCTTGACACCTT	CCAGGTCCCCGATCCCTCTAGCAT
FUT7	NM_004479	CCGCTTCTACCTGTCGTTTGA	GCGTTGCGCCAGAATTTCT	CGCGACTACATTACG

Analysis method

The Ct- values were calculated in the Quantstudio program, with the thresholds set according to plasmid controls. The average Ct- value of the triplicate of the sample were then exported to excel for analysis according to the delta- Ct- method. The Ct- value for the gene of interest

was linearized with the housekeeping gene RPL4 considered to assure the number of cells does not affect the Ct- value. The delta- Ct- method is used for qRT-PCR to calculate the relative gene expression. CT 40 was defined as relative unit 1.

GraphPad Prism 7.04 was used to create the graphs and t-tests were also performed in GraphPad Prism 7.04 using two- stage step- up method of Benjamini, Krieger and Yekutieli.

Ethics

In this study, embryonic stem cells have been used, HUM cells. These cells derivate from an aborted fetus and were isolated prior to the need for further ethical permits. Thus, no ethical permits were needed for our study.

Results

Our aim was to find out if expression of fucosyltransferase genes in Zika virus infected cells was increased. Other studies have demonstrated the importance of the E-protein for the Zika virus and other Flavivirus, but the contributing glycans, and which glycosyltranferases are involved in the synthesis of the essential N-linked glycan, is unknown (1, 33). Fucosyltranferases have earlier been shown to be important for spread of other viruses (41). By using qRT-PCR we analyzed cellular mRNA extracted from cell cultures in three batches (A549 cells and HUM cells) that we either successfully infected with Zika virus or mock-infected.

The resulting CT- values, based on the average of the sample triplicate analyzed, were calculated and set in relative numbers, taking RPL4 into account, a housekeeping gene that is direct proportional to the number of cells.

Time course of Zika virus RNA content in infected HUM and A549 cells

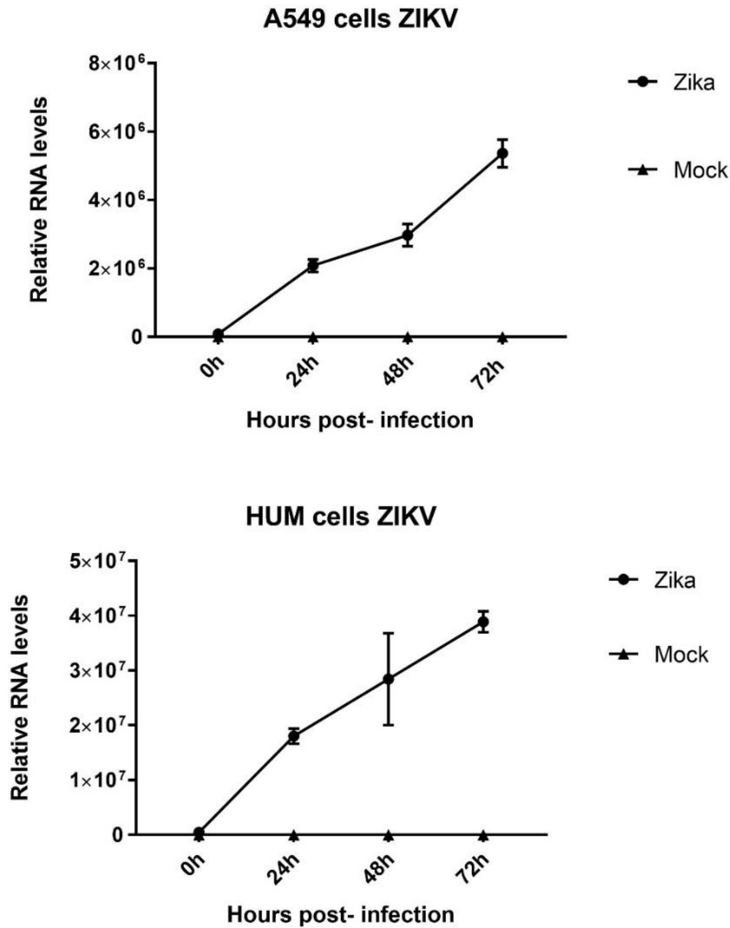

Fig. 1 Expression of ZIKV RNA at different time points in HUM and A549 cells infected with ZIKV or mock- infected. Error bars show the standard deviation.

Cell lysis and RNA extraction of intracellular ZIKV RNA content was analyzed by qRT-PCR which confirmed successful infection (Fig.1). Both cell cultures showed low initial amounts that linearly increased at the different measure hours post- infection, confirming a successful infection.

The mock infected cells, treated in parallel to the infection but without the addition of virus, served as negative control, showing as expected none, or negligible amount of ZIKV RNA content.

Gene expression of FUT2 and FUT4 in A549 cells and in HUM cells

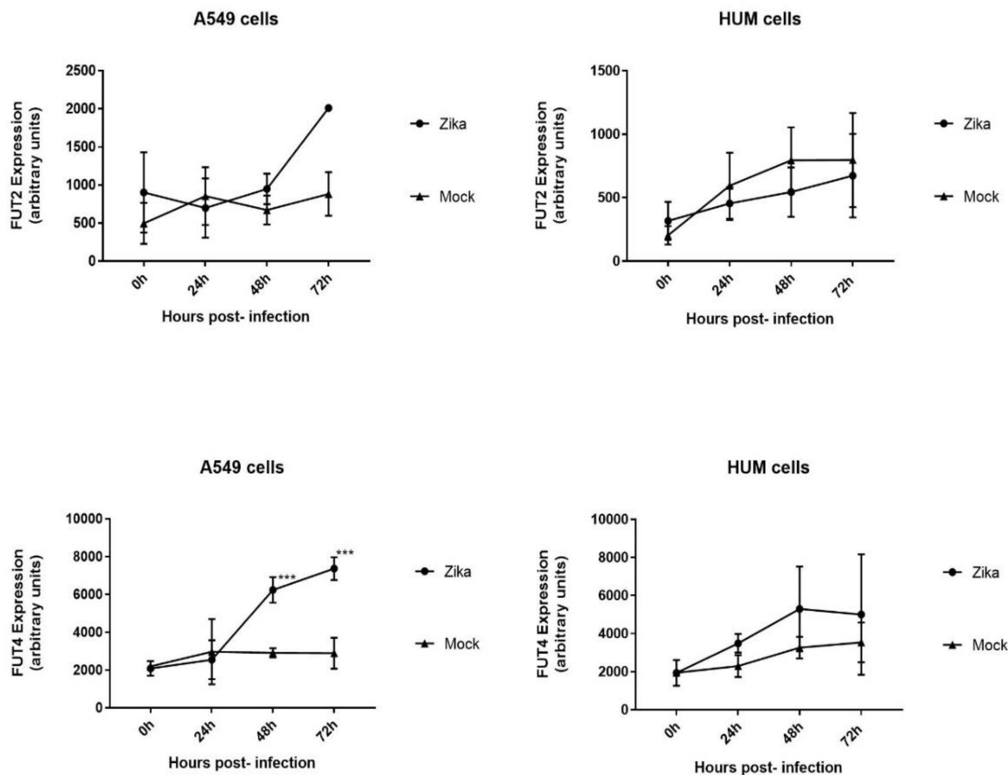

*Fig. 2. RNA expression of fucosyltransferase 2 and 4 in A549 and HUM cells at different timepoints post infection. Error bars show the standard deviation. *** P < 0,001.*

The expression of FUT4 in A549 cells (Fig.2) was increased at 48 and 72 hours post infection. T-test conducted on FUT4 expression in Zika virus infected A549 versus mock-infected A549 cells resulted in P- values 0.00035 48 hours post- infection and 0.00014 72 hours post- infection, thus showing significant increases. We saw no increase in ZIKV infected HUM cells.

There was a trend of increased FUT2 expression in A549 cells (Fig.2) at 72 hours post infection, compared to mock infected cells. However, this increase of FUT2 was not statically significant.

Gene expression of FUT1, FUT3, FUT5, FUT6 and FUT7 showed no difference between Zika infected and mock infected cells

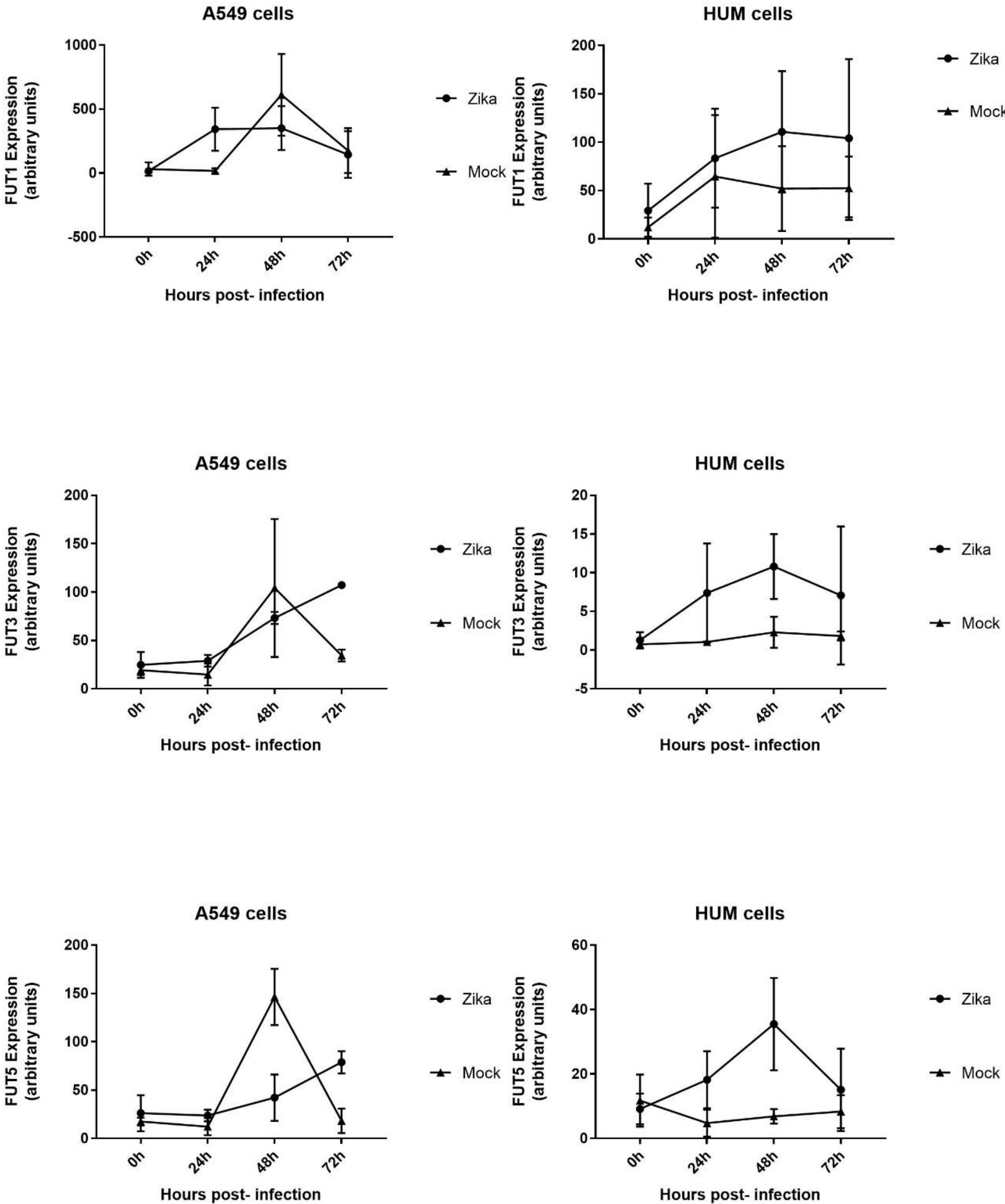

Fig. 3. RNA expression of fucosyltransferase genes 1, 3 and 5 in HUM and A549 cells at different timepoints post- infection. Error bars show the standard deviation. .

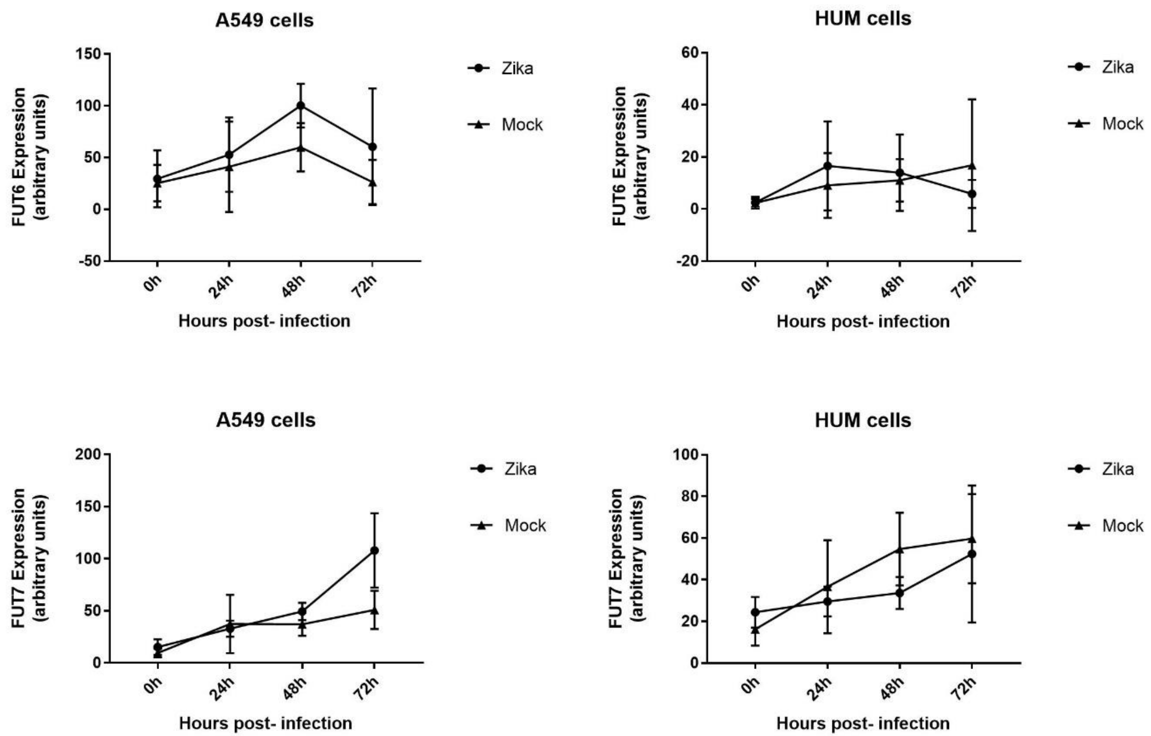

Fig. 4. RNA expression of fucosyltransferase gene 6 and 7 at different timepoints post-infection in A549 and HUM cells.

Analysis executed on ZIKV infected A549 cells and HUM cells regarding the genes FUT1, FUT3 and FUT5- 7 (Fig. 3-4) could not determine any difference in gene expression compared to mock infected cells. The relative expressions of these genes were also much lower than that of FUT2 and FUT4.

Discussion

The aim of this study was to investigate whether Zika virus infection alters the expression of fucosyltransferases. We have analyzed the expression of the fucosyltransferases genes 1 to 7 in two different human cell cultures that were successfully infected by the Zika virus. The conclusion is that Zika virus induces fucosyltransferase 4 expression in A549 cells but not in HUM cells.

A549 and HUM cells are of different cell lines and thereby exhibit different responses to infection. Due to intrinsic mechanisms fucosyltransferases are expressed in different levels also in non- infected cells. A549 are cells of an adenocarcinomic cell line and the HUM cells are embryonal fibroblasts. This means that different signaling pathways are active which accounts for different responses to cellular infection. The expression of a glycosyltransferase – as in the case with the differing expression of fucosyltransferase 4 we have demonstrated – is such an example. The response in vivo needs to be studied further and could also differ in different cell types. Since Zika virus infects different cell types in vivo, the glycosyltransferase expression as a result to viral infection might differ between different cell types.

In our study we used the Uganda strain, which lacks the N- glycan site on the E-protein. Thus, the effect of the N-glycan structure on viral pathogenesis cannot be investigated using the Uganda strain. However, cellular infection has been shown to be similar in both strains with or without the N- glycan. Other studies have shown no difference in infectivity or viral production in A549 cells between the Uganda strain and the South American strain. (42, 43). As we have demonstrated, even strains lacking the N- glycan site induce the expression of fucosyltransferase 4 in A549 infected cells. Since the primary difference between the two

strains is the N- glycan site, the viral induction of the fucosyltransferase gene seen in the Uganda strain would likely be seen in the South American strain as well (23). However, for further studies regarding the structure and function of the N- glycan, newer South American strains are necessary.

The main function of fucosyltransferase 4, which we have demonstrated to be increased in Zika virus infected A549 cells, is to synthesize Lewis^{x} by adding the needed fucose to the carbohydrate structure (29, 31, 44). One function of the Lewis^{x} is to bind to proteins and one of the most described such structure is DC- SIGN. DC-SIGN is a receptor expressed on dendritic cells and can bind to either N- glycan with high mannose or to the carbohydrate Lewis^{x}. (39). The E-protein of the Flaviviruses Dengue virus and West- Nile virus can bind to DC-SIGN with glycans. Cellular infectivity by West- Nile virus is heavily reduced when DC-SIGN- antibodies are present (20, 36, 37). It has also been demonstrated that the Zika virus can bind to DC-SIGN (38). Furthermore, fucoses are part of the viral binding sites (45, 46). Exactly how important DC-SIGN is for Flavi virus infectivity is not known.

Since Flavi virus and Zika virus can bind to DC-SIGN, the induction of fucosyltransferase 4 in Zika virus infected A549 cells which we have demonstrated, might lead to the needed synthesis of Lewis^{x} on the E-protein. However, this is not possible to conclude based on our study.

Other studies have demonstrated the importance of the N- glycan site of the Zika E-protein, which when deleted results in decreased infectivity of the central nervous system and viral production. Similar studies in the other Flavivirus West-Nile virus and Tick- borne encephalitis have also demonstrated the importance of the N-glycan site of the E- protein (34, 35). The exact role and which carbohydrates constituting the N- glycan of the E- protein is unknown (1). It is however evident that the N- glycan of the E-protein is important for

neuroinvasiveness, since the older strains lack the N-glycan and are not neuroinvasive. The induction of fucosyltransferase 4 in Zika virus infected cells might be a link in the pathogenesis.

The weaknesses of our study lie in the fact that we do not know what the increase of fucosyltransferase expression leads to and that our results are based on cell cultures. Future studies need to focus on the effects these increased expressions lead to by studying infected cells and the virions. The structure of the N- glycan needs to be clarified to conclude whether the increased expression of fucosyltransferases results in fucoses and or Lewis^{x}- antigens on the N- glycan of the E-protein. This needs to be conducted in the American/Asian strain. Also, the role of fucosyltransferases should be investigated in other Zika strains. Since the infectivity of the newer strains are more efficient in the CNS than in older ones, this differing mechanism is of interest. Determination of this mechanism and the responsible structures could be of interest in developing a treatment or a vaccine for Zika virus.

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Populärvetenskaplig sammanfattning

Zikaviruset upptäcktes första gången år 1947 men gick länge under radarn då det bara smittade ett fåtal människor. Först under 2000-talet började Zikaviruset orsaka stora utbrott, bl a i Brasilien. Det fick stor uppmärksamhet när fall kunde konstateras där foster hos gravida kvinnor som smittats med Zika viruset utvecklade för små hjärnor, mikrocefali. Orsaken till att Zikaviruset nu kunde smitta många fler låg i att det hade muterat och dess E-protein, ett protein som sitter på virusets yta, hade fått nya sätt att få sockermolekyler påkopplade. Det är oklart vilka sockermolekyler detta är, men det står fast att dessa är av stor vikt för viruset för att kunna infektera. Man har kunnat visa på experiment i möss att dessa sockermolekyler är nödvändiga. Om man tar bort platsen där de nya sockermolekyler kan bli påkopplade på E-proteinet, blir inte mössen infekterade i och med att de inte kan binda till cellen. Vi har i vår studie undersökt hur uttrycket av fucosyltransferasgener förändras i infekterade celler. Fucosyltransferasgener kodar för protein som kopplar på sockermolekylen fukos på sockermolekyler. Alla kroppens celler har samma DNA och gener, men alla gener uttrycks inte. De gener som uttrycks är det DNA som omskrivs till RNA, som sedan används som ritning för att tillverka ett protein – t ex en fucosyltransferas som i vårt fall. Zikaviruset skulle kunna påverka en infekterad cell till att uttrycka fucosyltransferasgener mer. Detta skulle bero på att fukos är viktig för att Zikaviruset ska kunna binda till celler och infektera dem.
För att ta reda på om det fanns RNA för genen använde vi en metod som heter kvantitativ PCR för att ta reda på hur mycket av genen som uttrycks. Metoden bygger på att gener i ett provrör kopieras och med hjälp av att molekyler som kan utsöndra ljus binder till genen som undersöks, kan en kamera i PCR- maskinen mäta intensiteten på ljuset som genen utstrålar. Ju fler kopior som finns av en gen, desto starkare blir därför ljuset och vi kan på så vis ta reda på

hur mycket av genen som uttrycks.

Vi jämförde Zikavirusinfekterade celler med celler som inte var infekterade.

Vi upptäckte att fucosyltransferasgenen 4 uttrycktes i högre grad i Zikavirusinfekterade celler jämfört med celler som inte var infekterade. Vi vet inte vad detta leder till, men möjligtvis kan det användas av viruset för att koppla på fukos på exempelvis E-proteinet, vilket skulle kunna vara ett led i hur viruset sprider sig i kroppen. I nästa steg behövs E-proteinets exakta struktur tas reda på, för att påvisa vilka sockermolekyler som finns på det.

References

1. Mossenta M, Marchese S, Poggianella M, Slon Campos JL, Burrone OR. Role of N-glycosylation on Zika virus E protein secretion, viral assembly and infectivity. Biochem Biophys Res Commun. 2017;492(4):579-86.
2. Macnamara FN. Zika virus: a report on three cases of human infection during an epidemic of jaundice in Nigeria. Trans R Soc Trop Med Hyg. 1954;48(2):139-45.
3. Petersen LR, Jamieson DJ, Powers AM, Honein MA. Zika Virus. N Engl J Med. 2016;374(16):1552-63.
4. Weaver SC, Costa F, Garcia-Blanco MA, Ko AI, Ribeiro GS, Saade G, et al. Zika virus: History, emergence, biology, and prospects for control. Antiviral Res. 2016;130:69-80.
5. Hennessey M, Fischer M, Staples JE. Zika Virus Spreads to New Areas - Region of the Americas, May 2015-January 2016. MMWR Morb Mortal Wkly Rep. 2016;65(3):55-8.
6. Gulland A. Zika virus is a global public health emergency, declares WHO. BMJ. 2016;352:i657.
7. Gubler DJ. The global emergence/resurgence of arboviral diseases as public health problems. Arch Med Res. 2002;33(4):330-42.
8. Hills SL, Russell K, Hennessey M, Williams C, Oster AM, Fischer M, et al. Transmission of Zika Virus Through Sexual Contact with Travelers to Areas of Ongoing Transmission - Continental United States, 2016. MMWR Morb Mortal Wkly Rep. 2016;65(8):215-6.
9. Musso D, Nhan T, Robin E, Roche C, Bierlaire D, Zisou K, et al. Potential for Zika virus transmission through blood transfusion demonstrated during an outbreak in French Polynesia, November 2013 to February 2014. Euro Surveill. 2014;19(14).
10. Duffy MR, Chen TH, Hancock WT, Powers AM, Kool JL, Lanciotti RS, et al. Zika virus outbreak on Yap Island, Federated States of Micronesia. N Engl J Med. 2009;360(24):2536-43.
11. Brasil P, Pereira JP, Jr., Moreira ME, Ribeiro Nogueira RM, Damasceno L, Wakimoto M, et al. Zika Virus Infection in Pregnant Women in Rio de Janeiro. N Engl J Med. 2016;375(24):2321-34.
12. . !!! INVALID CITATION !!! {}.
13. Cao-Lormeau VM, Blake A, Mons S, Lastere S, Roche C, Vanhomwegen J, et al. Guillain-Barre Syndrome outbreak associated with Zika virus infection in French Polynesia: a case-control study. Lancet. 2016;387(10027):1531-9.
14. Carteaux G, Maquart M, Bedet A, Contou D, Brugieres P, Fourati S, et al. Zika Virus Associated with Meningoencephalitis. N Engl J Med. 2016;374(16):1595-6.
15. Mecharles S, Herrmann C, Poullain P, Tran TH, Deschamps N, Mathon G, et al. Acute myelitis due to Zika virus infection. Lancet. 2016;387(10026):1481.
16. Fleming-Dutra KE, Nelson JM, Fischer M, Staples JE, Karwowski MP, Mead P, et al. Update: Interim Guidelines for Health Care Providers Caring for Infants and Children with Possible Zika Virus Infection--United States, February 2016. MMWR Morb Mortal Wkly Rep. 2016;65(7):182-7.

17. Mlakar J, Korva M, Tul N, Popovic M, Poljsak-Prijatelj M, Mraz J, et al. Zika Virus Associated with Microcephaly. N Engl J Med. 2016;374(10):951-8.
18. Franca GV, Schuler-Faccini L, Oliveira WK, Henriques CM, Carmo EH, Pedi VD, et al. Congenital Zika virus syndrome in Brazil: a case series of the first 1501 livebirths with complete investigation. Lancet. 2016;388(10047):891-7.
19. de Paula Freitas B, de Oliveira Dias JR, Prazeres J, Sacramento GA, Ko AI, Maia M, et al. Ocular Findings in Infants With Microcephaly Associated With Presumed Zika Virus Congenital Infection in Salvador, Brazil. JAMA Ophthalmol. 2016.
20. Mukhopadhyay S, Kuhn RJ, Rossmann MG. A structural perspective of the flavivirus life cycle. Nat Rev Microbiol. 2005;3(1):13-22.
21. Lorenz IC, Allison SL, Heinz FX, Helenius A. Folding and dimerization of tick-borne encephalitis virus envelope proteins prM and E in the endoplasmic reticulum. J Virol. 2002;76(11):5480-91.
22. Allison SL, Schalich J, Stiasny K, Mandl CW, Kunz C, Heinz FX. Oligomeric rearrangement of tick-borne encephalitis virus envelope proteins induced by an acidic pH. J Virol. 1995;69(2):695-700.
23. May M, Relich RF. A Comprehensive Systems Biology Approach to Studying Zika Virus. PLoS One. 2016;11(9):e0161355.
24. Faye O, Freire CC, Iamarino A, Faye O, de Oliveira JV, Diallo M, et al. Molecular evolution of Zika virus during its emergence in the 20(th) century. PLoS Negl Trop Dis. 2014;8(1):e2636.
25. Routhu NK, Byrareddy SN. Host-Virus Interaction of ZIKA Virus in Modulating Disease Pathogenesis. J Neuroimmune Pharmacol. 2017;12(2):219-32.
26. Berg J.M TJL, Stryer L. . Biochemistry 7th edition. New York: W.H Freeman and Company; 2012.
27. Breton C, Oriol R, Imberty A. Conserved structural features in eukaryotic and prokaryotic fucosyltransferases. Glycobiology. 1998;8(1):87-94.
28. Becker DJ, Lowe JB. Fucose: biosynthesis and biological function in mammals. Glycobiology. 2003;13(7):41R-53R.
29. de Vries T, Knegtel RM, Holmes EH, Macher BA. Fucosyltransferases: structure/function studies. Glycobiology. 2001;11(10):119R-28R.
30. Ma B, Simala-Grant JL, Taylor DE. Fucosylation in prokaryotes and eukaryotes. Glycobiology. 2006;16(12):158R-84R.
31. Markine-Goriaynoff N, Gillet L, Van Etten JL, Korres H, Verma N, Vanderplasschen A. Glycosyltransferases encoded by viruses. J Gen Virol. 2004;85(Pt 10):2741-54.
32. Han X, Bushweller JH, Cafiso DS, Tamm LK. Membrane structure and fusion-triggering conformational change of the fusion domain from influenza hemagglutinin. Nat Struct Biol. 2001;8(8):715-20.
33. Annamalai AS, Pattnaik A, Sahoo BR, Muthukrishnan E, Natarajan SK, Steffen D, et al. Zika Virus Encoding Non-Glycosylated Envelope Protein is Attenuated and Defective in Neuroinvasion. J Virol. 2017.
34. Whiteman MC, Li L, Wicker JA, Kinney RM, Huang C, Beasley DW, et al. Development and characterization of non-glycosylated E and NS1 mutant viruses as a potential candidate vaccine for West Nile virus. Vaccine. 2010;28(4):1075-83.
35. Yoshii K, Yanagihara N, Ishizuka M, Sakai M, Kariwa H. N-linked glycan in tick-borne encephalitis virus envelope protein affects viral secretion in mammalian cells, but not in tick cells. J Gen Virol. 2013;94(Pt 10):2249-58.
36. Kim SY, Li B, Linhardt RJ. Pathogenesis and Inhibition of Flaviviruses from a Carbohydrate Perspective. Pharmaceuticals (Basel). 2017;10(2).

37. Navarro-Sanchez E, Altmeyer R, Amara A, Schwartz O, Fieschi F, Virelizier JL, et al. Dendritic-cell-specific ICAM3-grabbing non-integrin is essential for the productive infection of human dendritic cells by mosquito-cell-derived dengue viruses. EMBO Rep. 2003;4(7):723-8.
38. Hamel R, Dejarnac O, Wichit S, Ekchariyawat P, Neyret A, Luplertlop N, et al. Biology of Zika Virus Infection in Human Skin Cells. J Virol. 2015;89(17):8880-96.
39. Holla A, Skerra A. Comparative analysis reveals selective recognition of glycans by the dendritic cell receptors DC-SIGN and Langerin. Protein Eng Des Sel. 2011;24(9):659-69.
40. Geijtenbeek TB, Kwon DS, Torensma R, van Vliet SJ, van Duijnhoven GC, Middel J, et al. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. Cell. 2000;100(5):587-97.
41. Hiraiwa N, Yabuta T, Yoritomi K, Hiraiwa M, Tanaka Y, Suzuki T, et al. Transactivation of the fucosyltransferase VII gene by human T-cell leukemia virus type 1 Tax through a variant cAMP-responsive element. Blood. 2003;101(9):3615-21.
42. Gong D, Zhang TH, Zhao D, Du Y, Chapa TJ, Shi Y, et al. High-Throughput Fitness Profiling of Zika Virus E Protein Reveals Different Roles for Glycosylation during Infection of Mammalian and Mosquito Cells. iScience. 2018;1:97-111.
43. Fontes-Garfias CR, Shan C, Luo H, Muruato AE, Medeiros DBA, Mays E, et al. Functional Analysis of Glycosylation of Zika Virus Envelope Protein. Cell Rep. 2017;21(5):1180-90.
44. Mollicone R, Cailleau A, Oriol R. Molecular genetics of H, Se, Lewis and other fucosyltransferase genes. Transfus Clin Biol. 1995;2(4):235-42.
45. van Liempt E, Bank CM, Mehta P, Garcia-Vallejo JJ, Kawar ZS, Geyer R, et al. Specificity of DC-SIGN for mannose- and fucose-containing glycans. FEBS Lett. 2006;580(26):6123-31.
46. Kambara C, Nakamura T, Furuya T, Nishiura Y, Kawakami A, Ichinose K, et al. Increased sialyl Lewis(x) antigen-positive cells mediated by HTLV-1 infection in peripheral blood CD4(+) T lymphocytes in patients with HTLV-1-associated myelopathy. J Neuroimmunol. 2002;125(1-2):179-84.